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Heterogeneity of chromatin subunits in vitro and location of histone H1.

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ABSTRACT

Chromatin subunits ("nucleosomes") which were purified by sucrose gradient centrifugation of a staphylococcal nuclease digest of chromatin have been studied. We found that such a preparation contains nucleosomes of two discrete types which can be separated from each other by polyacrylamide gel electrophoresis. Nucleosome of the first type contains all five histones and a DNA segment of approximately 200 base pairs long, whereas nucleosome of the second type lacks histone H1 and its DNA segment is approximately 170 base pairs long, i.e., about 30 base pairs shorter than the DNA segment of the nucleosome of the first type. Purified dimer of the nucleosome also can be fractionated by gel electrophoresis into three discrete bands which correspond to dinucleosomes containing two molecules of histone H1, one and no H1. These and related findings strongly suggest that the H1 molecule is bound to a short (approximately 30 base pairs) terminal stretch of the nucleosomal DNA segment which can be removed by nuclease (possibly in the form of H1-DNA complex) without any significant disturbance of main structural features of the nucleosome.

INTRODUCTION

Recent experimental findings strongly suggest that the structure of chromatin is based on a linear array of globular chromatin subunits which were called "nucleosomes" /1-18/. A sufficiently mild treatment of the chromatin with staphylococcal nuclease results in formation of a soluble mixture of separate nucleosomes (we shall call them mononucleosomes), their dimers and higher oligomers which can be separated from each other by centrifugation in a sucrose gradient /4,7,13/. Peaks of mono- and dinucleosomes in a sucrose gradient are usually sharp /7/ and therefore suggest a high degree of homogeneity of the corresponding deoxyribonucleoprotein (DNP) particles. We found however, that a preparation of purified

mononucleosomes is actually a mixture of DNP particles of two discrete structurally different types which can be separated from each other by polyacrylamide gel electrophoresis. The mononucleosome of the first type contains all five histones whereas the mononucleosome of the second type lacks histone H1 and besides this its DNA segment is approximately 30 base pairs shorter than the DNA segment of the nucleosome of the first type. Similar heterogeneity exists in preparations of the dinucleosome and probably also in the case of higher oligomers of the nucleosome.

These and related findings strongly suggest that the H1 molecule is bound to a short terminal stretch of the nucleosomal DNA segment. This DNA stretch (about 30 base pairs long) can be removed by nuclease (presumably in the form of H1-DNA complex) without any significant unfolding of DNA in the remaining nucleosomal DNP particle.

MATERIALS AND METHODS

Preparation of chromatin. All operations were carried out at 0–5°C. Chromatin was prepared from mouse Ehrlich ascites tumor cells /7,19/. The procedure included isolation of nuclei, washes of the nuclei in 0.15 M NaCl, 2 mM $MgCl_2$, 5 mM triethanolamine (TEA)-HCl, pH 7.6 (pH of buffers was measured at 18–20°C), then in 0.30 M NaCl, 2 mM $MgCl_2$, 5 mM TEA-HCl, pH 7.6 and finally in 5 mM TEA-HCl, pH 7.6. The chromatin gel obtained was gently suspended in 5 mM TEA-HCl, pH 7.6 and thereafter purified by centrifugation through a layer of 1.7 M sucrose, 5 mM TEA-HCl, pH 7.6. To facilitate handling of DNP preparations a labelled chromatin was used in the majority of experiments. Mice carrying Ehrlich tumor cells were injected intraperitoneally with a mixture of /Me-^{3}H/thymidine and a hydrolysate of Chlorella ^{14}C-proteins or with /Me-^{3}H/thymidine alone followed by isolation of the labelled chromatin /19/.

Nuclease digestion of chromatin and sucrose gradient centrifugation of digests. The chromatin gel (1.5 mg of DNA per ml) was treated with staphylococcal nuclease (3.5 µg/ml) in 1 mM $CaCl_2$, 1 mM TEA-HCl, pH 7.6 for 5–7 min at 37°C as described previously /7/. The digestion was stopped by addition

of 50 mM Na-EDTA, pH 7.6 to a final concentration of 2 mM followed by chilling of the sample in an ice bath. From 70 to 80% of the total DNP was solubilized under these conditions (that is, was not pelleted upon centrifugation at 10,000 g for 5 min) and about 5% of the total DNA was rendered acid--soluble as a result of nuclease treatment. From 15 to 25% of the total DNP in the digest was present in the form of mononucleosomes, most of the other DNP particles being oligonucleosomes /7/. The nuclease digest (20 ml) was layered onto 600 ml of a linear 10-40% sucrose gradient in 1 mM Na-EDTA, 1 mM TEA-HCl, pH 7.6 which was prepared in the Ti14 zonal rotor (Beckman). The rotor was centrifuged at 45,000 rpm for 22 hr at 3°C. Under these conditions the nucleosomes, their dimers and partially their trimers could be completely separated from each other, whereas the larger oligomers of the nucleosome were pelleted onto the wall of the zonal rotor /13/. Fractions which contained separated mono-, di- and trinucleosomes were dialysed overnight against 1 mM TEA-HCl, pH 7.6 in order to partially remove sucrose and thereafter concentrated by ultrafiltration in the Amicon apparatus to a final concentration of about 1.5 mg of DNA per ml. No detectable loss of the DNP occured during ultrafiltration.

It should be noted that chromatin isolated from mouse Ehrlich ascites tumor cells apparently did not contain endogeneous DNase or proteinase activities as was checked by appropriate controls (polyacrylamide gel electrophoresis and sucrose gradient centrifugation were used to monitor degradation of histones and of DNA, respectively). Staphylococcal nuclease used in this work (Schwarz/Mann) also did not contain proteinase impurities as was shown by similar control experiments.

Polyacrylamide gel electrophoresis of DNP. Concentrated DNP samples (from 5 to 30 µl) were loaded directly onto 5% polyacrylamide gels (0.6 cm internal diameter, 7 cm long) with an acrylamide:methylene-bisacrylamide ratio of 30:1. The buffer in the electrode vessels and in the gels was 2 mM Na--EDTA, 10 mM TEA-HCl, pH 7.6. Preelectrophoresis was carried out at 50 v (2 mA per gel) for 1 hr followed by electrophore-

sis of the DNP at 100 v (approximately 3 mA per gel) for 1.5 hr. Bromphenol blue was used as a mobility marker. The gels were stained with ethidium bromide (3 μg/ml in 2 mM Na-EDTA, pH 7.6 for 20 min), thereafter photographed without destaining through an orange filter under an ultraviolet illumination and finally stained for protein with Coomassie Brilliant Blue. Densitometer tracings of the Coomassie-stained gels were obtained with the use of a Joyce-Loeble microdensitometer. In some instances the unstained gels after electrophoresis of $^{14}C,^{3}H$-DNP samples were cut into 2 mm-thick slices, incubated with the NCS solubilizer (Amersham) and thereafter counted with toluene-PPO-POPOP in the computerized SL-40 counter (Intertechnique). In some experiments electrophoresis of the DNP was carried out in slab gels (12x12x0.15 cm).

Polyacrylamide gel electrophoresis of proteins in the presence of SDS. Ethidium bromide-stained individual DNP bands (1-2 mm in thickness) were cut from the gel and incubated for 20 min at 50°C in 2% SDS, 1% 2-mercaptoethanol, 10% glycerol, 60 mM Tris-HCl, pH 6.8. Thereafter each slice was placed onto a top of 15% polyacrylamide gel and the SDS-gel electrophoresis was carried out in the system of Laemmli as described by Bonner and Pollard /20/. Gels were incubated overnight in 50% CCl_3COOH followed by staining with 0.25% Coomassie in 50% CCl_3COOH for 2 hr. In some experiments the electrophoretic system and protocol of staining were those described by Bhorjee and Pederson /21/.

Polyacrylamide gel electrophoresis of DNA. DNA from the total mononucleosome preparation was isolated by a double extraction with a mixture of chloroform and isoamyl alcohol (24:1) in the presence of 3 mM Na-EDTA, 1% SDS and 1 M NaCl. The aqueous phase was dialysed against water at 4°C and liophilized; the liophilized material was dissolved in sample buffer and thereafter subjected to gel electrophoresis (see below). To isolate DNA from the electrophoretically separated mononucleosomes the corresponding unstained DNP bands in a slab gel were cut with a razor blade (a small strip of a slab gel was stained with ethidium bromide to locate the required bands). The gel slices were gently homogenized in 5 volumes

of 0.5% SDS, 3 mM Na-EDTA, pH 7.6 using a hand homogenizer. The slurry was stirred overnight at ~ 20°C after which the pieces of gel were removed by centrifugation at 10,000 g for 15 min. The extract was made 1.5 M in NaCl, cooled to ~ 4°C and the precipitated SDS was removed by centrifugation at 10,000 g for 10 min. The supernatant contained 98-100% of the original DNA but virtually no proteins which were co-precipitated with SDS /22,23/. The supernatant was extracted once with an equal volume of chloroform-isoamyl alcohol (24:1) and processed further as described above for the total mononucleosomal DNA. In some experiments isolated DNP bands were electroeluted from the gel as described previously /24/. Two electrophoretic systems were used and gave similar results. The first system was identical to that used for gel electrophoresis of the DNP except that longer gels were employed (15 cm) and the concentration of polyacrylamide was 6%. The second electrophoretic system was that of Peacock and Dingman /25/ with the use of 6% polyacrylamide slab gels.

Hin restriction endonuclease DNA fragments generated from SV40 DNA were used as molecular weight standards /26,27/. Hin restriction endonuclease was a gift from Dr. V.I.Tanyashin, SV40 DNA was kindly given to us by Dr.P.M.Chumackov.

RESULTS

Fractionation of nuclease digest of chromatin. Double-labelled chromatin which was prepared frpm mouse Ehrlich ascites tumor cells was treated with staphylococcal nuclease as described in Methods. The digest which contained mononucleosomes, their dimers and higher oligomers was fractionated by sucrose gradient centrifugation in the Ti14 zonal rotor (Fig. 1). Peaks of the mononucleosomes, their dimers and trimers (the latter peak was contaminated by the admixture of higher oligomers of the nucleosome) were concentrated by ultrafiltration and thereafter subjected to a polyacrylamide gel electrophoresis. To avoid aggregation of histone H1-containing DNP particles in usually used high ionic strength electrophoretic buffers we carried out the electrophoresis at a relatively low ionic strength of solution (~ 0.015) (Fig.2). The gels were stained for DNA with ethidium bromide (Fig.2a-d) or al-

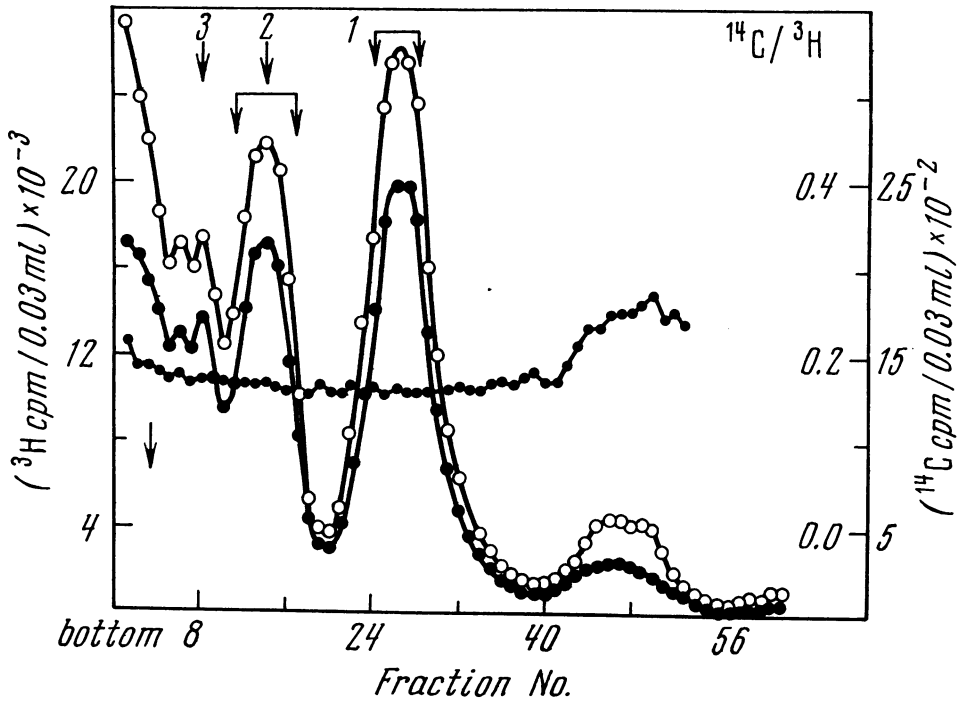

Fig.1. Sucrose gradient centrifugation of nuclease digest of chromatin in Ti14 zonal rotor. ●, ^{3}H (DNA); ○, ^{14}C (protein); ●, $^{14}C/^{3}H$. Arrows in the upper left of the graph indicate peaks of mono-, di- and trinucleosomes. An arrow in the lower left indicates a 2.1 M sucrose shelf. See Methods for detail.

ternatively, with a protein-specific stain Coomassie Brilliant Brue (Fig.2e-h). The electrophoretic pattern of the total nuclease digest of the chromatin is shown in Fig.2a (ethidium staining) and in Fig.2h (Coomassie staining). In both gels and especially clearly in the gel h one can see two widely separated discrete bands with the highest mobility, then three bands with an intermediate mobility and also a significant amount of the material near the origin of the gel. It should be noted that a similar picture was observed when the total nuclease digest of isolated, unwashed nuclei was analysed by gel electrophoresis (data not shown). Electrophoretic analy-

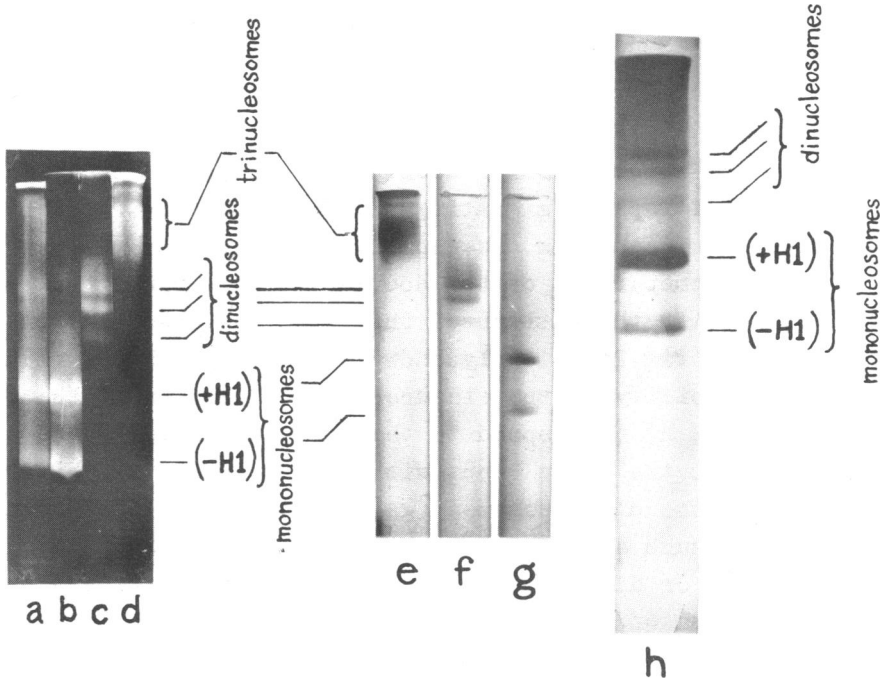

Fig.2. Polyacrylamide gel electrophoresis of DNP.
Gels a-d were stained with ethidium bromide; gels e-h were stained with Coomassie.
a and h - total nuclease digests of the chromatin (from different experiments).
b and g - purified mononucleosomes.
c and f - purified dinucleosomes (the relative content of the third, most rapidly migrating dinucleosome was usually lower than that of the other two dinucleosomes).
d and e - trinucleosomes with an admixture of higher oligomers of the nucleosome (see Figure 1).

sis of the purified mono-, di- and trinucleosomes permitted us to identify individual bands in the electrophoretic pattern of the total nuclease digest of the chromatin. Figure 2 shows the electrophoretic patterns of the purified mononucleosomes (gel b - ethidium staining, gel g - Coomassie staining), of the purified dinucleosomes (gel c - ethidium staining, gel f - Coomassie staining) and of the partially purified trinucleosomes (gel d - ethidium staining, gel e - Coomassie stain-

ing). Although the trinucleosome preparation (gels d and e) was significantly heterogeneous, no separation into discrete bands was achieved so far and therefore we shall consider below only mono- and dinucleosomes. Furthermore, it should be noted that for a yet unknown reason the relative content of the third, most rapidly migrating dinucleosome (Fig.2 c,f,h) significantly varied from one experiment to another.

A simple comparison of the above-mentioned electrophoretic patterns with the pattern of the total nuclease digest of the chromatin (Fig.2h) clearly shows that the two bands with the highest mobility in the electrophoretic pattern of the total digest (gel h) correspond to the mononucleosomes (gels b and g) and that the three bands with an intermediate mobility correspond to the dinucleosomes (gels c and f). To understand the nature of such a "discrete" heterogeneity within either mononucleosome or dinucleosome preparations we determined protein composition of mono- and dinucleosomes and also the lengths of their DNA segments.

Protein composition of mono- and dinucleosomes. To determine a protein composition of mono- and dinucleosomes which were resolved by gel electrophoresis we cut individual DNP bands after the electrophoresis (see Fig.2) and incubated them in an SDS-containing buffer. Each incubated polyacrylamide slice was placed onto a top of an SDS-containing polyacrylamide gel and a second electrophoretic run was carried out as described in Methods. As can be seen from Fig.3, the mononucleosome with the highest mobility in Fig.2 lacks histone H1 (Fig.3b), whereas the other mononucleosome contains a full complement of histones (Fig.3a). Similar analysis of the dinucleosomes showed that the dinucleosome with the lowest mobility in Fig.2c,f contained a full complement of histones (Fig.3e). Although the more rapidly migrating dinucleosome also contained all five histones (Fig.3d), the relative content of histone H1 in it as determined from densitometer tracings of the corresponding gels, was approximately two times lower than that in the first dinucleosome (Fig.3e; cf. Fig. 3d). Finally, the third dinucleosome with the highest mobility (Fig. 2c,f,h) lacked histone H1 (Fig.3c).

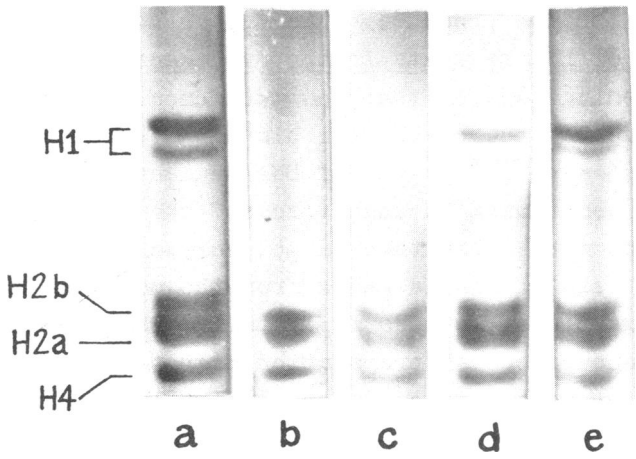

Fig.3. Protein composition of mono- and dinucleosomes.
Proteins from:
(a) more slowly migrating mononucleosome (see Fig.2b,g);
(b) rapidly migrating mononucleosome;
(c) most rapidly migrating dinucleosome (see Fig.2h);
(d) dinucleosome with an intermediate mobility;
(e) third dinucleosome with the lowest mobility (see Methods for detail).

It should be noted that fixation of the DNP with formaldehyde /7,19/ did not result in any significant change of the electrophoretic patterns as compared with the pattern given by unfixed DNP (Fig.2), providing that free formaldehyde was removed from DNP samples by dialysis before the electrophoresis.

Figure 4 shows that the percentage of the H1-depleted mononucleosomes in the total mononucleosome preparation is gradually increased during nuclease digestion of the chromatin. This phenomenon is not due to a degradation of H1 during incubation of the chromatin with staphylococcal nuclease since control electrophoretic analyses of histones which were isolated from the nuclease-treated chromatin, failed to detect any significant degradation of histone H1. It should be noted that the H1-depleted mononucleosome was the major DNP particle which was solubilized during nuclease digestion of chromatin before addition of EDTA i.e., in the presence of $CaCl_2$. The majority of H1-containing DNP particles remained

insoluble due to the presence of Ca^{2+} ions (data not shown).

Lengths of DNA segments in mononucleosomes. It was shown in the preceeding section that the two mononucleosomes differ from each other by the content of histone H1. Is there any difference between the lengths of DNA segments of the H1-depleted and H1-containing mononucleosomes? The answer is yes as can be seen from the results of a polyacrylamide gel electrophoretic analysis of DNA isolated from the mononucleosomes of each type (Fig.5; see Methods for detail). DNA isolated from the total mononucleosomal preparation migrated in the gel in two bands with close but nonidentical mobilities (Fig.5a).

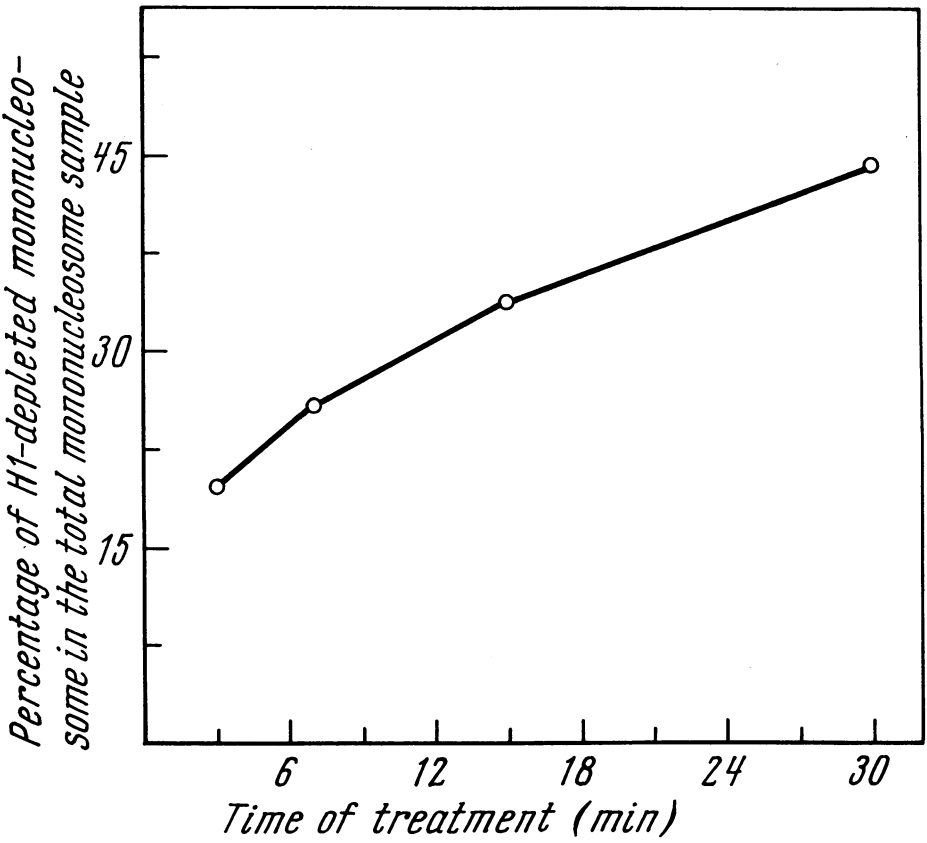

Fig.4. Percentage of H1-depleted mononucleosome in the total mononucleosome preparation as a function of time of nuclease treatment.
The points were calculated from the gel electrophoregrams of total digests of the $^{14}C,^{3}H$-chromatin which were counted as described in Methods.

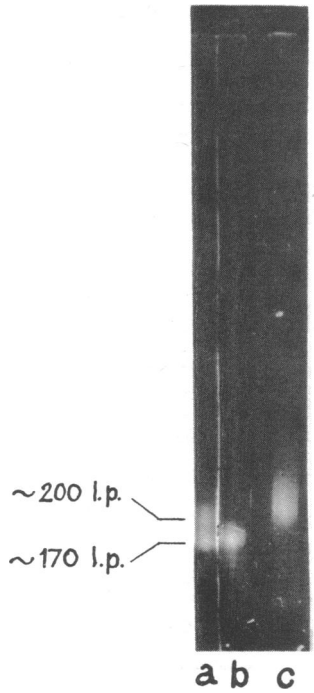

Fig.5. Polyacrylamide gel electrophoresis of mononucleosomal DNA.
(a) Total mononucleosomal DNA.
(b) DNA isolated from the histone H1-depleted mononucleosome.
(c) DNA isolated from the mononucleosome which contained a full complement of histones. See Methods for detail.

These mobilities corresponded to a DNA length of approximately 170 base pairs for the faster migrating band in Fig.3a and to approximately 200 base pairs for the slower migrating band as determined by comparison with the mobilities of Hin restriction endonuclease fragments generated from SV40 DNA /26, 27/. Figure 5b shows that the DNA segment from the histone H1-depleted mononucleosome migrates in the gel in one band with the mobility corresponding to a length of approximately 170 base pairs, whereas the DNA segment from the mononucleosome containing all five histones has a length of approximately 200 base pairs (Fig.3c). Thus the two mononucleosomes differ from each other not only by the presence or absence of

histone H1 but also by the length of their DNA segments. So far we did not carry out similar analysis of the dinucleosomal DNA. However, a general similarity of histone H1-dependent heterogeneity pattern of the mononucleosomes to that of the dinucleosomes (see Fig.2) suggests that there exists a corresponding microheterogeneity of the lengths of dinucleosomal DNA segments.

It should be noted that Noll /4/ previously observed a doublet of DNA electrophoretic bands which corresponded to mononucleosomes in a deproteinized nuclease digest of rat liver chromatin. It is now clear from the results described above that this DNA doublet corresponds to the doublet of mononucleosomes one of which lacks H1, whereas the other one contains a full complement of histones. Implications of these findings will be discussed below.

Subnucleosomal DNP particles. We have found previously that a small but significant amount of the DNP in a mild nuclease digest of the chromatin sedimented more slowly than mononucleosomes (ref.7; see also Figure 1, fractions 42-50). We call such DNP particles "subnucleosomes". Subnucleosomes can be detected not only by sucrose gradient centrifugation but also by gel electrophoresis of the total nuclease digest of the chromatin (Fig.6). It should be noted that the formation of subnucleosomes under our experimental conditions is apparently not due to a partial degradation of some histones (see above) in contrast to the trypsin-induced subnucleosomal DNP particles in the experiments of Weintraub /28/. Detailed analysis of composition and origin of the subnucleosomes will be presented elsewhere (Bakayev et al., in preparation).

DISCUSSION

The main result of this work is that mono- and dinucleosomes and probably also higher nucleosome oligomers which are produced during nuclease digestion of the chromatin are heterogeneous with regard to the content of histone H1 and also to the lengths of their DNA segments. Since the mononucleosomal DNP particle apparently contains one H1 molecule /3,7/ one can predict the existence of only two types of mononucleosomes (namely, mononucleosomes containing and lacking H1) if

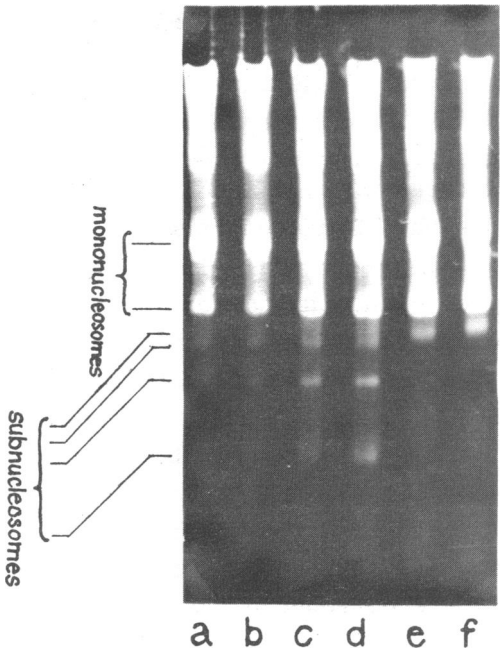

Fig.6. Slab gel electrophoresis of DNP of total nuclease digest of chromatin.

The gels were overexposed to reveal minor subnucleosomal bands.

(a) Chromatin was treated with nuclease for 3 min as described in Methods (10 µg/ml of enzyme) followed by a direct loading of the DNP onto a slab gel.

b,c,d,e and f - the same as a but enzymic treatment was for 5,8,15,30 and 60 min, respectively.

Notice that two rapidly migrating subnucleosomes in b,c, and d dissapear upon additional digestion (e and f), probably as a result of a further degradation.

a heterogeneity with regard to H1 is considered. Indeed, we observed only two discrete bands upon polyacrylamide gel electrophoresis of the purified mononucleosomes (see Fig.2). Similarly, one can predict three types of dinucleosomes differing from each other by the content of histone H1 (dinucleosomes containing two H1 molecules, one and no H1). In accordance with this prediction we observed three discrete bands of dinucleosomes upon gel electrophoresis (Fig.2). The same line of reasoning predicts an increasing number of subspecies

upon an increase of a length of an oligomer of the nucleosome.

The DNA segment of the H1-depleted mononucleosome is approximately 30 base pairs shorter than that of the mononucleosome which contains all five histones (see Results). Since no intermediate structures e.g., a mononucleosome with a full complement of histones but with a shorter DNA segment were observed upon the electrophoretic analysis of the mononucleosome preparations one can conclude that a loss of histone H1 and a shortening of the nucleosomal DNA segment occur simultaneously during nuclease treatment of the chromatin. Thus it is possible that histone H1 is removed in a complex with a DNA fragment of about 30 base pairs in length. Indeed, we have found an H1-DNA complex among subnucleosomes which were present in a mild nuclease digest of the chromatin (unpublished data). To check directly the above suggestion one needs preparative amounts of the electrophoretically separated subspecies of the mono- and dinucleosomes. Treatment of such preparations with nuclease should then permit one to establish directly precursor-product relationships between different mono- and dinucleosomes and also between subnucleosomal DNP particles. Work in this direction is now in progress.

Independently of the above considerations the data obtained in this work strongly suggest that histone H1 is located at the periphery of the nucleosomal DNP particle. Application of cross-linking reagents for studies on histone-histone interactions in chromatin has shown that histone H1 predominantly forms oligomers which almost exclusively consist of H1 /20, 29-31/. This result is not incompatible with the above-mentioned interpretation since oligomers of the nucleosome apparently form a higher-order coil in solution /32/. In such coils H1 molecules which are bound to each terminal stretch of the nucleosomal DNA segment may be brought into sufficiently close contact to be cross-linked with each other.

Finally, it should be noted that the existence of the dinucleosome which completely lacks histone H1 in a total nuclease digest of chromatin (see Results) suggests that not every H1-binding site on nucleosomal DNA segments is associated with H1.

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